(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 334 301 A1

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 89105059.3

(51) Int. Cl.⁴ C12N 7/00 , C12N 15/00 , A61K 35/76 , G01N 33/569

(22) Date of filing: 21.03.89

(30) Priority: 21.03.88 US 170515

(43) Date of publication of application: 27.09.89 Bulletin 89/39

(84) Designated Contracting States: AT BE CH DE ES FR GB GR IT LI LU NL SE

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(54) Recombinant retroviruses.

(57) Recombinant retroviruses carrying a vector construct capable of preventing, inhibiting, stabilizing or reversing infectious, cancerous or auto-immune diseases are disclosed. More specifically the recombinant retroviruses of the present invention are useful for (a) stimulating a specific immune response to an antigen or a pathogenic antigen; (b) inhibiting a function of a pathogenic agent, such as a virus; and (c) inhibiting the interaction of an agent with a host cell receptor. In addition, eucaryotic cells infected with, and pharmaceutical compositions containing such a recombinant retrovirus are disclosed. Various methods for producing recombinant retroviruses having unique characteristics, and methods for producing transgenic packaging animals or insects are also disclosed.

EP 0 334 301 A1

RECOMBINANT RETROVIRUSES

Technical Field

The present invention relates generally to retroviruses, and more specifically, to recombinant retroviruses which are capable of delivering vector constructs to susceptible target cells. These vector constructs are typically designed to express desired proteins in target cells, for example, proteins which stimulate immunogenic activity or which are conditionally active in defined cellular environments.

Background of the Invention

Although bacterial diseases are, in general, easily treatable with antibiotics, very few effective treatments or prophylactic measures exist for many viral, cancerous, and other nonbacterial diseases, including genetic diseases. Traditional attempts to treat these diseases have employed the use of chemical drugs. In general, these drugs have lacked specificity, exhibited high overall toxicity, and thus have been therapeutically ineffective.

Another classic technique for treating a number of nonbacterial diseases involves the elicitation of an immune response to a pathogenic agent, such as a virus, through the administration of a noninfectious form of the agent, such as a killed virus, thereby providing antigens from the pathogenic agent which would act as an immunostimulant.

A more recent approach for treating viral diseases, such as acquired immunodeficiency syndrome (AIDS) and related disorders, involves blocking receptors on cells susceptible to infection by HIV from receiving or forming a complex with viral envelope proteins. For example, Lifson et al. (Science 232:1123-1127, 1986) demonstrated that antibodies to CD4 (T4) receptors inhibited cell fusion (syncytia) between infected and noninfected CD4 presenting cells in vitro. A similar CD4 blocking effect using monoclonal antibodies has been suggested by McDougal et al. (Science 231:382-385, 1986). Alternatively, Peri et al. (Proc. Natl. Acad. Sci. USA 83:9254-9258, 1986) have reported the use of synthetic peptides to bind T4 receptors and block HIV infection of human T-cells, while Lifson et al. (J. Exp. Med. 164:2101, 1986) have reported blocking both syncytia and virus-T4 cell fusion by using a lectin which interacts with a viral envelope glycoprotein, thereby blocking it from being received by CD4 receptors.

A fourth, recently suggested technique for inhibiting a pathogenic agent, such as a virus which transcribes RNA, is to provide antisense RNA which complements at least a portion of the transcribed RNA, and binds thereto, so as to inhibit translation (To et al., Mol. Cell. Biol. 6:758, 1986).

However, a major shortcoming of the techniques described above is that they do not readily lend themselves to control as to the time, location or extent to which the drug, antigen, blocking agent or antisense RNA are utilized. In particular, since the above techniques required exogenous application of the treatment agent (i.e., exogenous to the sample in an in vitro situation), they are not directly responsive to the presence of the pathogenic agent. For example, it may be desirable to have an immunostimulant expressed in increased amounts immediately following infection by the pathogenic agent. In addition, in the case of antisense RNA, large amounts would be required for useful therapy in an animal, which under current techniques would be administered without regard to the location at which it is actually needed, that is, at the cells infected by the pathogenic agent.

As an alternative to exogenous application, techniques have been suggested for producing treatment agents endogenously. More specifically, proteins expressed from viral vectors based on DNA viruses, such as adenovirus, simian virus 40, bovine papilloma, and vaccinia viruses, have been investigated. By way of example, Panicali et al. (Proc. Natl. Acad. Sci. USA 80:5364, 1983) introduced influenza virus hemagglutinin and hepatitis B surface antigens into the vaccinia genome and infected animals with the virus particles produced from such recombinant genes. Following infection, the animals acquired immunity to both the vaccinia virus and the hepatitis B antigen.

However, a number of difficulties have been experienced to date with viral vectors based on DNA viruses. These difficulties include (a) the production of other viral proteins which may lead to pathogenesis or the suppression of the desired protein; (b) the capacity of the vector to uncontrollably replicate in the host, and the pathogenic effect of such uncontrolled replication; (c) the presence of wild-type virus which may lead to viremia; and (d) the transitory nature of expression in these systems. These difficulties have virtually precluded the use of viral vectors based on DNA viruses in the treatment of viral, cancerous, and other nonbacterial diseases, including genetic diseases.

Due to the nontransitory nature of their expression in infected target cells, retroviruses have been suggested as a useful vehicle for the treatment of genetic disease (for example, see F. Ledley, The Journal of Pediatrics 110:1, 1987). However, in view of a number of problems, the use of retro-

viruses in the treatment of genetic diseases has not been attempted. Such problems relate to (a) the apparent need to infect a large number of cells in inaccessible tissues (e.g., brain); (b) the need to cause these vectors to express in a very controlled and permanent fashion; (c) the lack of cloned genes; (d) the irreversible damage to tissue and organs due to metabolic abnormalities; and (e) the availability of other partially effective therapies in certain instances.

In addition to genetic diseases, other researchers have contemplated using retroviral vectors to treat nongenetic diseases (see, for example, EP 243,204 - Cetus Corporation; Sanford, J. Theor. Biol.130:469, 1988; Tellier et al., Nature 318:414, 1985; and Bolognesi et al., Cancer Res. 45:4700, 1985).

Tellier et al. suggested protecting T-cell clones by apparently infecting stem cells with "defective" HIV having a genome which could express antisense RNA to HIV RNA. Bolognesi et al. have suggested the concept of generating a nonvirulent HIV strain to infect stem cells so that T4 cells generated therefrom would carry interfering, nonvirulent forms of virus and thereby protect those cells from infection by virulent HIV. However, it would appear that the "attenuated" or "defective" HIV viruses used in both of the foregoing papers could reproduce (i.e., are not replication defective) such that the resulting viruses could infect other cells, with the possibility of an increased risk of recombination with previously present HIV or other sequences, leading to loss of attenuation. Non-nonreplicative forms would necessitate a defective helper or packaging line for HIV. However, since the control of HIV gene expression is complex, such cells have to date not been constructed. Furthermore, as the infecting attenuated or defective virus is not chimeric (a "nonchimeric" retrovirus being one with substantially all of its vector from the same retrovirus species), even if they were made replication defective, for example by deletion from their genomes of an essential element, there still exists a significant possibility for recombination within the host cells with resultant production of infectious viral particles.

Although Sanford (J. Theor. Biol. 130:469, 1988) has also proposed using a genetic cure for HIV, he notes that due to the potential that exists for creating novel virulent viruses via genetic recombination between natural AIDS virus and therapeutic retroviral vectors carrying anti-HIV genes, retroviral gene therapy for AIDS may not be practical. Similarly, while McCormick & Kriegler (EP 243,204 A2) have proposed using retroviral vectors to deliver genes for proteins, such as tumor necrosis factor (TNF), the techniques they describe suffer from a number of disadvantages.

Summary of the Invention

Briefly stated, the present invention is directed, in part, toward methods for (a) stimulating a specific immune response, either humoral or cell-mediated, to an antigen or pathogenic antigen; (b) inhibiting a function of a pathogenic agent, such as a virus; and (c) inhibiting the interaction of an agent with a host cell receptor, through the use of recombinant retroviruses.

More specifically, within one aspect of the present invention, a method for stimulating a specific immune response is provided, comprising infecting susceptible target cells with recombinant retroviruses carrying a vector construct that directs the expression of an antigen or modified form thereof in infected target cells. Where an immune response is to be stimulated to a pathogenic antigen, the recombinant retrovirus is preferably designed to express a modified form of the antigen which will stimulate an immune response and which has reduced pathogenicity relative to the native antigen. An immune response can also be achieved by transferring to an appropriate immune cell (such as a T lymphocyte) the gene for the specific T- cell receptor which recognizes the antigen of interest in the context of an appropriate MHC molecule or for an immunoglobulin which recognizes the antigen of interest.

In the particular case of disease caused by HIV infection, where immunostimulation is desired, the antigen generated from the recombinant retroviral genome is of a form which will elicit either or both an HLA class I- or class II-restricted immune response. In the case of HIV envelope antigen, for example, the antigen is preferably selected from gp 160, gp 120, and gp 41, which have been modified to reduce their pathogenicity. In particular, the antigen selected is modified to reduce the possibility of syncytia. Antigens from other HIV genes, such as gag, pol, vif, nef, etc., may also provide protection in particular cases.

In another aspect of the present invention, methods for inhibiting a function of a pathogenic agent necessary for disease, such as diseases caused by viral infections, cancers or immunological abnormalities, are disclosed. Such inhibition is accomplished by means which include expressing a palliative that is toxic for a diseased cell, expressing a palliative that selectively inhibits the expression or the effects of pathogenic genes, expressing antisense RNA, or by inserting a sequence into a pathogenic genome so as to disrupt its function.

More specifically, the present invention provides recombinant retroviral genomes which expresses a defective structural protein of a pathogenic agent, leading to inhibition of assembly of the pathogenic agent, e.g., expression of a defec-

one which conveys resistance to an otherwise cytotoxic drug. The cells are then exposed to a selecting agent, preferably the cytotoxic drug, which enables identification of those cells which express the selectable protein at a critical level (i.e., in the case of a cytotoxic drug, by killing those cells which do not produce a level of resistance protein required for survival).

Preferably, in the technique briefly described above, the expressions of both the selectable and primary genes is controlled by the same promoter. In this regard, it may be preferable to utilize a retroviral 5' LTR, in order to maximize titre of a recombinant retrovirus from packaging cells, this technique is first used to select packaging cells expressing high levels of all the required packaging proteins, and then is used to select which of these cells, following transfection with the desired proviral construct, produce the highest titres of the recombinant retrovirus.

Techniques are also provided for packaging of vector constructs by means not involving the use of packaging cells. These techniques make use of DNA viruses such as baculovirus, adenovirus, or vaccinia virus, preferably adenovirus. These viruses are known to express relatively high levels of proteins from exogenous genes provided therein. For such DNA virus vectors, recombinant DNA viruses can be produced by in vivo recombination in tissue culture between viral DNA and plasmids carrying retroviral or retroviral vector genes. The resultant DNA viral vectors carrying either sequences coding for retroviral proteins or for retroviral vector RNA are purified into high titre stocks. Alternatively, the constructs can be constructed in vitro and subsequently transfected into cells which provide in trans viral functions missing from the DNA vectors. Regardless of the method of production, high titre (10^7 to 10^{11} units/ml) stocks can be prepared that will, upon infection of susceptible cells, cause high level expression of retroviral proteins (such as gag, pol, and env) or RNA retroviral vector genomes, or both. Infection of cells in culture with these stocks, singly or in combination, will lead to high-level production of retroviral vectors, if the stocks carry the viral protein and viral vector genes. This technique, when used with adenovirus or other mammalian vectors, allows the use of primary cells (e.g., from tissue explants or cells such as WI38 used in production of vaccines) to produce recombinant retroviral vectors.

In an alternative to the foregoing technique, recombinant retroviruses are produced by first generating the gag:pol and env proteins from a cell line infected with the appropriate recombinant DNA virus in a manner similar to the preceding techniques, except that the cell line is not infected with a DNA virus carrying the vector construct. Subsequently, the proteins are purified and contacted with the desired viral vector RNA made in vitro, transfer RNA (tRNA), liposomes, and a cell extract to process the env protein into the liposomes, such that recombinant retroviruses carrying the viral vector RNA are produced. Within this technique, it may be necessary to process the env protein into the liposomes prior to contacting them with the remainder of the foregoing mixture. The gag-pol and env proteins may also be made after plasmid mediated transfection in eukaryotic cells, in yeast, or in bacteria.

The technique for producing recombinant retroviruses which can be targeted for preselected cell lines utilizes recombinant retroviruses having an env gene comprised of a cytoplasmic segment of a first retroviral phenotype, and an extracellular binding segment exogenous to the first retroviral phenotype. The binding segment is from a second viral phenotype or from another protein with desired binding properties which is selected to be expressed as a peptide which will bind to the desired target.

Techniques for integrating a retroviral genome at a specific site in the DNA of a target cell involve the use of homologous recombination, or alternatively, the use of a modified integrase enzyme which will recognize a specific site on the target cell genome. Such site-specific insertion allows genes to be inserted at sites on the target cells' DNA, which will minimize the chances of insertional mutagenesis, minimize interference from other sequences on the DNA, and allow insertion of sequences at specific target sites so as to reduce or eliminate the expression of an undesirable gene (such as a viral gene) in the DNA of the target cell.

It will be appreciated that any of the above-described techniques may be used independently of the others in particular situations, or can be used in conjunction with one or more of the remainder of the techniques.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 depicts three different families of vectors used to produce HIV env and which may or may not have the selectable SV-Neo cassette inserted.

Figure 2 illustrates the HIV env expression levels seen in polyacrylamide gel electrophoresis of HIV env specific radioimmune precipitations of extracts of human Sup T1 cells transfected with the vectors shown. The markers are in kilodaltons, gp

the present invention provides such a stimulus.

By way of example, in the case of HIV-1 infections, patients develop antibodies specific for a variety of viral envelope-region determinants, some of which are capable of in vitro virus neutralization. Nevertheless, disease progression continues and the patients eventually succumb to the disease. Low-level CTL responses against infected patients' cells (Plata et al., Nature 328:348-351, 1987) and against target cells infected with recombinant vaccinia vectors expressing HIV gag, pol, or env (Walker et al., Nature 328:345-348, 1987; Walker et al., Science 240: 64-66, 1988) have been detected in some HIV-1 seropositive patients. In addition, it has recently been shown that murine as well as human CTL can be induced by autologous stimulator cells expressing HIV gp 120 via transfection (Langlade-Demoyan et al., J. Immunol. 141:1949, 1988). Improved CTL induction could be therapeutically advantageous to injected patients and provide effective preventive therapy to individuals under noninfectious conditions. HIV infection itself may not be producing adequate CTL response because other elements associated with HIV infection may prevent proper immune stimulation. In addition, it may be that stimulation of T-cells by infected cells is an interaction that leads to infection of the stimulated T-cells.

Example I describes procedures for constructing plasmids capable of generating retroviral vectors in packaging cells, which then lead to expression of HIV viral antigens.

EXAMPLE 1

Vectors Expressing HIV Antigens

A. Env Expression Vector (See Figure 1)

A 2.7 kb Kpn-Xho I DNA fragment was isolated from the HIV proviral clone BH10-R3 (for sequence see Ratner et al., Nature 313:277, 1985) and a ~400 bp Sal-Kpn I DNA fragment from IIIexE7deltaenv (a Bal31 deletion to nt. 5496) was ligated into the Sal I site in the plasmid SK⁺. From this clone, a 3.1 kb env DNA fragment (Xho I-Cla I) which also encodes rev, essential for env expression, was purified and ligated into a retroviral vector called pAFVXM (see Kriegler et al., Cell 38:483, 1984). This vector was modified in that the Bgl II site was changed by linker insertion to a Xho I site to facilitate cloning of the HIV env coding DNA fragment.

A dominant selectable marker gene comprised of a SV40 early promoter driving expression of neomycin phosphotransferase gene was inserted into the vector at the Cla I site to facilitate isolation of infected and transfected cell lines.

The Xho I site upstream from the ENV gene in the vector provides a convenient site to insert additional promoters into the vector construct as the RSV promoter, SV40 early or late promoter, the CMV immediate early (IE) promoter, human beta-actin promoter, and Moloney murine MLV SL3-3 promoter.

One such promoter, the CMV Immediate Early gene promoter, a 673 bp DNA fragment Hinc II to Eag I, results in a tenfold increase in ENV expression in a human T-cell line called Sup T1 when compared to the parental construct pAF ENV⁺ SV_2 Neo.

B. Gag Expression Vector:

A 2.5 kb Sac I-Eco RV DNA fragment was isolated from pBH10-R3 (see Ratner et al., op. cit.) and ligated into the Sac I-Sal I site of pUC31. pUC31 is derived from pUC19 with additional Xho I, Bgl II, Bsst II and Nco I sites inserted between the Eco R1 and Kpn I sites of the poly linker. However, this construct contained the major splice donor (SD) site from HIV and thus could be problematic in virus generation. The SD site was removed by subcloning a 70 bp Rsa I-Cla I fragment with a 2.1 kb Cla I-Bam H1 DNA fragment into the Hinc II-Bam H1 site of SK⁺. The Bam H1 site was converted into a Cla I site by linker insertion. This construct was designated SK⁺ gag protease SD delta.

The 2.5 kb Xho I-Cla I DNA fragment from SK⁺ gag protease SD delta was inserted into the Xho I Cla I sites of the vector pAFVXM just as described above.

These plasmids, when placed in a suitable packaging cell, expressed a retroviral vector construct which contains a packaging signal. The packaging signal directed packaging of the vector construct into a capsid and envelope along with all further proteins required for viable retroviral particles. The capsid, envelope, and other proteins are preferably produced from one or more plasmids containing suitable genomes placed in the packaging cell. Such genomes may be proviral constructs, which in a simple case may merely have the packaging signal deleted. As a result, only the vector will be packaged. Suitable packaging or packaging cell lines, and the genome necessary for accomplishing such packaging, are described in Miller et al. (Mol. Cell. Bio. 6:2895, 1986), which is incorporated herein by reference. As described by

EXAMPLE 3

sCD4 Vector

1. A 1.7 kb Eco R1 - Hind III DNA fragment from pMV7.T4 (Maddon et al., Cell 47:333, 1986) was blunt-end ligated to the Hinc II site of Sk

2. A universal translation termination sequence containing an Xba I site was inserted into the Nhe I site of the CD4 fragment.

3. The 1.7 kb Xho I-Cla I fragment was excised and cloned into the Xho I - Cla I site of pXFVXM. These vector plasmids can be used to generate infectious vector particles, as described in Example 1.

Such infectious blocking vectors, when put into human T-cell lines in culture can inhibit the spread of HIV infections. Preparation, concentration and storage of infectious retroviral vector preparations is as for the immunostimulant. Route of administration would also be the same, with doses about 10-fold higher. Another route which may be used is the aspiration of bone marrow, infection with retroviral vector and return of this infected marrow (Gruber et al., Science 230:1057, 1985) to the patient. Since the marrow replication will amplify the vector expression through cell replication, doses in the range of the immunostimulant can be used (10^5 - 10^6/kg body weight).

In any case, the efficacy of the treatment can be assayed by measuring the usual indicators of disease progression, including antibody level, viral antigen production, infectious HIV levels, or levels of non-specific infections.

III. Expression of Palliatives

Techniques similar to those described above can be used to produce recombinant retroviruses with vector constructs which direct the expression of an agent (or "palliative") which is capable of inhibiting a function of a pathogenic agent or gene. Within the present invention, "capable of inhibiting a function" means that the palliative either directly inhibits the function or indirectly does so, for example, by converting an agent present in the cells from one which would not normally inhibit a function of the pathogenic agent to one which does. Examples of such functions for viral diseases include absorption, replication, gene expression, assembly, and exit of the virus from infected cells. Examples of such functions for cancerous diseases include cell replication, susceptibility to external signals (e.g., contact inhibition), and lack of production of anti-oncogene proteins.

(i) Inhibitor Palliatives

In one aspect of the present invention, the vector construct directs the expression of antisense RNA (or complementary RNA) to RNA of a pathogenic virus, such as HIV (or a pathogenic gene, such as an oncogene), to thereby inhibit its replication or pathogenesis. Such expression may either be essentially continuous or in response to the presence in the cell of another agent associated with the pathogenic condition (an "identifying agent"). Alternatively, the expression may be tissue-specific due either to targeting of vector entry or to tissue-specific control sequences in the vector.

In one embodiment, retroviral viruses which express RNA complementary to key pathogenic gene transcripts (for example, a viral gene product or an activated cellular oncogene) can be used to inhibit translation of that transcript into protein, such as the inhibition of translation of the HIV tat protein. Since expression of this protein is essential for viral replication, cells containing the vector would be resistant to HIV replication.

In a second embodiment, where the pathogenic agent is a single-stranded virus having a packaging signal, RNA complementary to the viral packaging signal (e.g., an HIV packaging signal when the palliative is directed against HIV) is expressed, so that the association of these molecules with the viral packaging signal will, in the case of retroviruses, inhibit stem loop formation or tRNA primer binding required for proper encapsidation or replication of the retroviral RNA genome.

In a third embodiment, a retroviral vector may be introduced which expresses a protein that interferes with the pathogenic state. In the case of HIV one example is a mutant tat protein which lacks the ability to transactivate expression from the HIV LTR and interferes (in a transdominant manner) with the normal functioning of tat protein. Such a mutant has been identified for HTLV II tat protein ("XII Leu^{3}" mutant; see Wachsman et al., Science 235:674, 1987). A mutant transrepressor tat should inhibit replication much as has been shown for an analogous mutant repressor in HSV-1 (Friedmann et al., Nature 335:452, 1988).

Such a transcriptional repressor protein may be selected for in tissue culture using any viral-specific transcriptional promoter whose expression is stimulated by a virus-specific transactivating protein (as described above). In the specific case of HIV, a cell line expressing HIV tat protein and the HSVTK gene driven by the HIV promoter will die in

the presence of ACV. However, if a series of mutated tat genes are introduced to the system, a mutant with the appropriate properties (i.e., represses transcription from the HIV promoter in the presence of wild-type tat) will grow and be selected for. The mutant gene can then be reisolated from these cells. A cell line containing multiple copies of the conditionally lethal vector tat system may be used to assure that surviving cell clones are not caused by endogenous mutations in these genes. A battery of randomly mutagenized tat genes are then introduced into these cells using a "rescuable" retroviral vector (i.e., one that expresses the mutant tat protein and contains a bacterial origin of replication and drug resistance marker for growth and selection in bacteria). This allows a large number of random mutations to be evaluated and permits facile subsequent molecular cloning of the desired mutant cell line. This procedure may be used to identify and utilize mutations in a variety of viral transcriptional activator-viral promoter systems for potential antiviral therapies.

In a fourth embodiment, the HSVTK gene product is used to more effectively metabolize potentially antiviral nucleoside analogues, such as AZT or ddC. The HSVTK gene is expressed under the control of a constitutive macrophage or T-cell-specific promoter and introduced into these cell types. AZT (and other nucleoside antivirals) must be metabolized by cellular mechanisms to the nucleotide triphosphate form in order to specifically inhibit retroviral reverse transcriptase and thus HIV replication (Furman et al., Proc. Natl. Acad. Sci. USA 83:8333-8337, 1986). Constitutive expression of HSVTK (a nucleotide and nucleotide phosphate kinase with very broad substrate specificity) results in more effective metabolism of these drugs to their biologically active nucleotide trionosphate form. AZT or ddC therapy will thereby be more effective, allowing lower doses, less generalized toxicity, and higher potency against productive infection. Additional nucleosice analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleosice and nucleotide kinases are not phosphorylated, will be made more efficacious. A description of this method is set forth in Example 4.

EXAMPLE 4

Vectors Designed to Potentiate the Antiviral Effect of AZT and Analogues

I. All of the following retroviral vectors are based on the "N2" vector (see Keller et al., Nature 318:149-154, 1985). Consequently, 5' and 3' Eco R1 LTR fragments (2.8 and 1.0 kb, respectively) were initially subcloned into plasmids containing polylinkers (into SK+ to give pN2R5[+ =]; into pUC31 to give p31N2R5[+ -] and p31N2R3[+ -] to facilitate vector construction. In one case, a 1.2 kb Cla I/Eco R1 5' LTR fragment was subcloned into the same sites of an SK+ vector to give pN2C. In another case, the 5' LTR containing a 6 bp deletion of the splice donor sequence was subcloned as a 1.8 kb Eco R1 fragment into pUC31 (p31N25delta-[+]). The coding region and transcriptional termination signals of HSV-1 thymidine kinase gene was isolated as a 1.8 kb Bgl II/Pvu II fragment from plasmid 322TK (3.5 kb Bam H1 fragment of HSVTK cloned into Bam H1 of pBR322) and cloned into Bgl II/Sma I-digested pUC31 (pUCTK). For constructs which require deletion of the terminator signals, pUCTK was digested with Sma I and Bam H1. The remaining coding sequences and sticky-end Bam H1 overhang were reconstituted with a double-stranded oligonucleotide made from the following oligomers:

5' GAG AGA TGG GGG AGG CTA ACT GAG 3'

and 5' GAT CCT CAG TTA GCC TCC CCC ATC TCT C 3'

forming the construct pTK delta A.

For diagnostic purposes, the oligos were designed to destroy the Sma I site while keeping its Ava I site without changing the translated protein.

The 0.6 kb HIV promoter sequences were cloned as a Dra I/Hind III fragment from pCV-1 (see Arya et al., Science 229:69-73, 1985) into Hinc II/Hind III-cut SK (SKHL).

A. Construction of TK-1 and TK-3 retroviral vectors (see Figure 6).

1. The 5 kb Xho I/Hind III 5' LTR and plasmid sequences were isolated from p31N2R5(+).

2. HSVTK coding sequences lacking transcriptional termination sequences were isolated as 1.2 kb Xho I/Bam H1 fragment from pTKdeltaA.

3. 3' LTR sequences were isolated as a 1.0 kb Bam H1/Hind III fragment from pN2R3(-)

4. The fragments from steps 1-3 were mixed, ligated, transformed into bacteria, and individual clones identified by restriction enzyme analysis (TK-1).

5. TK-3 was constructed by linearizing TK-1 with Bam H1, filling in the 5' overhang and blunt-end ligating a 5'-filled Cla I fragment containing the bacterial lac UV5 promoter, SV40 early promoter, plus Tn5 Neo' gene. Kanamycin-resistant clones were isolated and individual clones were screened

are). these two levels of specificity (viral integration-replication and tissue-specific transcriptional regulation) lead to preferential killing of tumor cells. Additionally, event-specific and tissue-specific promoter elements may be artificially combined such that the cytotoxic gene product is expressed only in cell types satisfying both criteria (e.g., in the example above, combined promoter elements are functional only in rapidly dividing liver cells). Transcriptional control elements may also be amplified to increase the stringency of cell-type specificity.

These transcriptional promoter:enhancer elements need not necessarily be present as an internal promoter (lying between the viral LTRs) but may be added to or replace the transcriptional control elements in the viral LTRs which are themselves transcriptional promoters, such that condition-specific transcriptional expression will occur directly from the modified viral LTR. In this case, either the condition for maximal expression will need to be mimicked in retroviral packaging cell lines (e.g., by altering growth conditions, supplying necessary transregulators of expression or using the appropriate cell line as a parent for a packaging line), or the LTR modification is limited to the 3' LTR U3 region, to obtain maximal recombinant viral titers. In the latter case, after one round of infection-integration the 3' LTR U3 is now also the 5' LTR U3 giving the desired tissue specific expression.

In a third embodiment, the proviral vector construct is similarly activated but expresses a protein which is not itself cytotoxic, and which processes within the target cells a compound or a drug with little or no cytotoxicity into one which is cytotoxic (a "conditionally lethal" gene product). Specifically, the proviral vector construct carries the herpes simplex virus thymidine kinase ("HSVTK") gene downstream and under the transcriptional control of an HIV promoter (which is known to be transcriptionally silent except when activated by HIV tat protein). Expression of the tat gene product in human cells infected with HIV and carrying the proviral vector construct causes increased production of HSVTK. The cells (either in vitro or in vivo) are then exposed to a drug such as acyclovir or its analogues (FIAU, FIAC, DHPG). These drugs are known to be phosphorylated by HSVTK (but not by cellular thymidine kinase) to their corresponding active nucleotide triphosphate forms (see, for example, Schaeffer et al., Nature 272:583, 1978). Acyclovir and FIAU triphosphates inhibit cellular polymerases in general, leading to the specific destruction of cells expressing HSVTK in transgenic mice (see Borrelli et al., Proc. Natl. Acad. Sci. USA 85:7572, 1988). Those cells containing the recombinant vector and expressing HIV tat protein are selectively killed by the presence of a specific dose of these drugs. In addition, an extra level of specificity is achieved by including in the vector the HIV rev protein, responsive CRS:CAR sequences. In the presence of the CRS sequence gene expression is suppressed, except in the presence of the CAR sequences and the rev protein. Example 5 provides an illustration of this technique.

EXAMPLE 5

Vector to Conditionally Potentiate the Toxic Action of ACV or its Analogues

Construction of Vectors

A. Construction of pKTVIHAX (see Figure 7).

1. A 9.2 kb Asu II/Xho I fragment was isolated from vector pN2 DNA.

2. A 0.6 kb Xho I/Bam H1 promoter fragment was isolated from plasmid pSKHL.

3. A 0.3 kb Bg III/Acc I and a 1.5 kb Acc I/Acc I fragment were purified from pUCTK.

4. The fragments from 1, 2 and 3 were ligated, transformed into bacteria, and appropriate Amp' clones of the given structure identified by restriction enzyme analysis.

B. Construction of pKTVIH-5 and pKTVIH5 Neo retroviral vectors (see Figure 8).

1. 4.5 kb 5' LTR and vector was isolated as an Xho I/Bam H1 fragment from vector p31N25delta(-).

2. The 1.0 kb 3' LTR was isolated as an Apa I/Bam H1 fragment from pN2R3(-) fragment.

3. The 0.6 kb HIV promoter element was isolated from pSKHL as an Apa I/Eco R1 fragment.

4. The HSVTK coding sequence and transcriptional termination sequences were isolated as 1.8 kb Eco R1/Sal I fragment from pUCTK.

5. The fragments from 1-4 were combined, ligated, transformed into bacteria, and clones of the given structure were identified by restriction enzyme analysis (pKTVIH-5).

6. pKTVIH5 Neo was constructed by linearizing pKTVIH5 with Cla I; mixing with a 1.8 kb Cla I fragment containing the bacterial lac UV5 promoter, SV40 early promoter, and Tn5 Neo' marker; ligating, transforming bacteria and selecting for

expression. In addition, multimerization of these sequences (i.e., rev-responsive "CRS-CAR" or tat-responsive "TAR" elements for HIV) could result in even greater specificity. It should be noted that this kind of conditional activation of an inactive precursor into an active product in cells may also be achieved using other viral vectors with a shorter term effect, e.g., adenovirus vectors.

Production, concentration and storage of vector preparations is as previously described. Administration is by direct in vivo administration as before or by ex corpore treatment of PBL and/or bone marrow. Doses will be at approximately the same levels as for Example 4. Targeting of viral vector infection will not be through the CD4 receptor, but may be accomplished through making vector particles with hybrid MLVenv-CD4 "envelope" proteins (see Section VII) to target gp 120 expressing cells (i.e., those infected with HIV). This inversion of the normal virus-receptor interaction in order to target virally infected cells can be used with all types of viruses.

In a similar manner to the preceding embodiment, the retroviral vector construct can carry a gene for phosphorylation, phosphoribosylation, ribosylation, or other metabolism of a purine- or pyrimidine-based drug. This gene may have no equivalent in mammalian cells and might come from organisms such as a virus, bacterium, fungus, or protozoan. An example of this would be the E. coli guanine phosphoribosyl transferase gene product, which is lethal in the presence of thioxanthine (see Besnard et al., Mol. Cell. Biol. 7 4139-4141, 1987). Conditionally lethal gene products of this type have potential application to many presently known purine- or pyrimidine-based anticancer drugs, which often require intracellular ribosylation or phosphorylation in order to become effective cytotoxic agents. The conditionally lethal gene product could also metabolize a nontoxic drug, which is not a purine or pyrimidine analogue, to a cytoxic form.

Mammalian viruses in general tend to have "immediate early" genes which are necessary for subsequent transcriptional activation from other viral promoter elements. Gene products of this nature are excellent candidates for intracellular signals (or "identifying agents") of viral infection. Thus, conditionally lethal genes from transcriptional promoter elements responsive to these viral "immediate early" gene products could specifically kill cells infected with any particular virus. Additionally, since the human α and β interferon promoter elements are transcriptionally activated in response to infection by a wide variety of nonrelated viruses, the introduction of vectors expressing a conditionally lethal gene product like HSVTK, for example, from these viral-responsive elements (VRE, could result in the destruction of cells infected with a variety of different viruses.

In a fourth embodiment, the recombinant retrovirus carries a gene specifying a product which is not in itself toxic, but when processed by a protease specific to a viral or other pathogen, is converted into a toxic form.

In a fifth embodiment, the retroviral construct may express a "reporting product" on the surface of the target cells in response to the presence of an identifying agent in the cells (such as HIV tat protein). This surface protein can be recognized by a cytotoxic agent, such as antibodies for the reporting protein or by cytotoxic T-cells. In a similar manner, such a system can be used as a detection system (see below) to simply identify those cells having a particular gene which expresses an identifying protein, such as the HIV tat gene.

Similarly, in a sixth embodiment, a surface protein could be expressed which would itself be therapeutically beneficial. In the specific case of HIV, expression of the human CD4 protein in HIV-infected cells may be beneficial in two ways:

1. Binding of CD4 to HIV env intracellularly could inhibit the formation of viable viral particles much as soluble CD4 has been shown to do for free virus, but without the problem of systematic clearance and possible immunogenicity, since the protein will remain membrane bound and is structurally identical to endogenous CD4 (to which the patient should be immunologically tolerant).

2. Since the CD4-HIV env complex has been implicated as a cause of cell death, additional expression of CD4 (in the presence of excess HIV-env present in HIV-infected cells) leads to more rapid cell death and thus inhibits viral dissemination. This may be particularly applicable to monocytes and macrophages, which act as a reservoir for virus production as a result of their relative refractility to HIV-induced cytotoxicity (which, in turn, is apparently due to the relative lack of CD4 on their cell surfaces).

(iii) Immune Down-Regulation

Specific down-regulation of inappropriate or unwanted immune response, such as in chronic hepatitis or in transplants of heterologous tissue such as bone marrow, can be engineered using anti-MHC class I genes, such as immune-suppressive viral genes. Group C adenoviruses Ad2 and Ad5 possess a 19 kd glycoprotein (gp 19) encoded in the E3 region of the virus. This gp 19 molecule binds to class I MHC molecules in the endoplasmic reticulum of cells and prevents terminal glycosylation and translation of class I MHC to the cell surface.

were derived as follows:

The retroviral backbone was derived from the construct pAFVXM (Krieger et al., Cell 38:384, 1984), which had been linearized using Xho I and Cla I. SV_2neo was obtained from the plasmid pKoneo (Hanahen, unpubl.) by isolation of the 1.8 kb Cla I fragment.

The HIV LTR was isolated as a 0.7 kb Hind III fragment from the plasmid pC15CAT (Arya et al., Science 229:69, 1985). Beta-gal was obtained from the plasmid pSP65 β-gal (Capko, pers. comm.) as a Hind III-Sma I fragment. A secreted form of human placental alkaline phosphatase was produced by introduction of a universal terminator sequence after amino-acid 489 of the cell surface form of alkaline phosphatase (as described by Berger et al., Gene 66:1, 1988). The secreted alkaline phosphatase gene was isolated as a 1.8 kb Hind III to Kpn I fragment. The CRS-CAR sequences from HIV env were obtained by isolating the 2.1 kb Kpn I to Bam H1 fragment from HTLVIIIB-BH10R3 (Fisher et al., Science 233:655: 1986). This fragment was inserted into pUC31 linearized by Bam HI, and Kpn I pUC31 is pUC19 (Yanisch-Perron et al., Gene 33:103, 1985) with extra Xho I, B-gl II, Bssh II and Nco I sites between the Eco RI and Kpn I sites of pUC19. The Bam H1 site of the resulting construct was converted to a Nco I site to allow resection of the CRS-CAR sequences by Nco I digestion. The SV40 t intron was obtained from pSVOL (de Wet et al., Mol. Cell. Biol. 7:725, 1987) as a 0.8 kb Nco I to Bam H1 fragment.

B. Indicator Cells and Retroviral Vectors

HUman T-cell (H-9, CEM and Sup T1) and monocyte (U-937) cell lines were obtained from ATCC, and maintained in RPM1 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin streptomycin.

The nonretroviral vectors were introduced into cell lines by electroporation using a Bio-Rad Gene Pulser. The cell lines were selected in G-418 (1 mg/ml) for 2-3 weeks to obtain stable G-418$_R$ cell lines, and then dilution cloned to obtain clonal cell lines.

The pAF vectors were transfected into the PA317 packaging cell line as a calcium phosphate precipitate (Wigler et al., Cell 16:777, 1979). The virus-producing PA317 cells were co-cultivated with human monocyte cell lines for 24 hours in the presence of polybrene, after which the suspension cells were removed and selected in G-418 and subcloned as above.

C. Assay

Stable cell lines were infected with HIV (HTLV IIIB) and the cells (β-gal) or media (alkaline phosphatase) assayed on a daily basis for 6 days post-infection.

β-Galactosidase Assay

Infected cells could be assayed by either: (i) in situ histochemical staining as described by MacGregor et al. Somatic Cell and Mol. Genetics 13:253, 1987); or (ii) by using cell extracts in a solution enzymatic assay with ONPG as a substrate (Norton and Coffin, Mol. Cel. Biol. 5:281, 1985).

Soluble Alkaline Phosphatase Assay

Medium was removed from infected cells, microfuged for 10 seconds, and then heated to 68°C for 10 minutes to destroy endogenous phosphatases. The medium was then microfuged for 2 minutes and an aliquot (10-50 µl) removed for assay. 100 µl of buffer (1 M diethanolamine, pH 9.8; 0.5 Mm $MgCl_2$; 10 mM L-homoarginine) was added and then 20 µl of 120 mM p-nitrophenylphosphate (in buffers) was added. The A_{405} of the reaction mixture was monitored using an automatic plate reader.

Figures 14 and 15 depict typical results of a time course of infection of Sup T1 cells using the alkaline phosphatase assay in the presence of varying concentrations of antiviral drugs. The "+" and "-" on day 6 indicate the presence or absence of syncytia.

The present invention provides a number of other techniques (described below) which can be used with the retroviral vector systems employed above, so as to enhance their performance. Alternatively, these techniques may be used with other gene-delivery systems.

V. Packaging Cell Selection

This aspect of the present invention is based, in part, upon the discovery of the major causes of low recombinant virus titres from packaging cells, and of techniques to correct those causes. Basically, at least five factors may be postulated as causes for low recombinant virus titres:

1. the limited availability of viral packaging proteins;

2. the limited availability of retroviral vector RNA genomes;

3. the limited availability of cell membrane for budding of the recombinant retroviruses;

Natl. Acad. Sci. USA 80:477, 1983) that has the SV40 promoter, the pBR322 ampicillin resistance and origin of replication and the SV40 poly A site. This gives pSVgp. pSVgpDHFR was made using the following fragments: the 3.6 kb Hind III to Sal I fragment from pSVgp containing the SV40 promoter plus MLV gag and some pol sequences; the 2.1 kb Sal I to Sca I fragment from pMLV-K with the rest of the pol gene, the 3.2 kb Xba I (Xba I filled-in) to Pst I fragment from pF400 with the DHFR gene plus poly A site, pBR322 origin and half the amoicillin resistance gene; the 0.7 kb Pst I to Hind III fragment from pBR322 with the other half of the ampicillin resistance gene. This gives pSVgp-DHFR. All these constructs are shown in Figure 7. These plasmids can be transfected into 3T3 cells or other cells and high levels of gag, pol or env obtained.

An additional method for accomplishing selection is to use a gene selection in one round and its antisense in a subsequent round. For example, gag-pol may be introduced into an HPRT-deficient cell with the HPRT gene and selected for the presence of this gene using that media which requires HPRT for the salvage of purines. In the next round, the antisense to HPRT could be delivered downstream to env and the cell selected in 6 thioguanine for the HPRT-deficient phenotype. Large amounts of antisense HPRT would be required in order to inactivate the HPRT gene transcripts, assuming no reversion occurred.

Envelope Substitutions

The ability to express gagpol and env function separately allows for manipulation of these functions independently. A cell line that expresses ample amounts of gagpol can be used, for example, to address questions of titre with regard to env. One factor resulting in low titres is the density of appropriate receptor molecules on the target cell or tissue. Given that env expression is from a separate unit, a variety of envelope genes (requiring different receptor proteins), such as xenotropic, polytropic, or amphotropic envs from a variety of sources, can be tested for highest titres on a specific target tissue. Furthermore, envelopes from nonmurine retrovirus sources can be used for pseudotyping a vector. In addition, hybrid envelopes (as described below) can be used in this system as well, to tailor the tropism (and effectively increase titres) of a retroviral vector. Conversely, a cell line that expresses ample amounts of a given envelope gene can be employed to address questions of titre with regard to gag and pol.

VI. Alternative Viral Vector Packaging Techniques

Two additional alternative systems can be used to produce recombinant retroviruses carrying the vector construct. Each of these systems takes advantage of the fact that the insect virus, baculovirus, and the mammalian viruses, vaccinia and adenovirus, have been adapted recently to make large amounts of any given protein for which the gene has been cloned. For example, see Smith et al. (Mol. Cell. Biol. 3:12. 1983; Piccini et al. (Meth. Enzymology. 153:545. 1987); and Mansour et al. (Proc. Natl. Acad. Sci. USA 82:1359. 1985).

These viral vectors can be used to produce proteins in tissue culture cells by insertion of appropriate genes into the viral vector and, hence, could be adapted to make retroviral vector particles.

Adenovirus vectors are derived from nuclear replicating viruses and can be defective. Genes can be inserted into vectors and used to express proteins in mammalian cells either by in vitro construction (Ballay et al., EMBO J. 4:3861. 1985) or by recombination in cells (Thummel et al., J. Mol. Appl. Genetics 1:435. 1982).

One preferred method is to construct plasmids using the adenovirus Major Late Promoter (MLP) driving: (1) gag-pol, (2) env, (3) a modified viral vector construct. A modified viral vector construct is possible because the U3 region of the 5' LTR, which contains the viral vector promoter, can be replaced by other promoter sequences (see, for example, Hartman, Nucl. Acids Res. 16:9345. 1988). This portion will be replaced after one round of reverse transcriptase by the U3 from the 3' LTR.

These plasmids can then be used to make adenovirus genomes in vitro (Ballay et al., op. cit.), and these transfected in 293 cells (a human cell line making adenovirus E1A protein), for which the adenoviral vectors are defective, to yield pure stocks of gag, pol, env and retroviral vector carried separately in defective adenovirus vectors. Since the titres of such vectors are typically 10^7-10^{11} ml, these stocks can be used to infect tissue culture cells simultaneously at high multiplicity. The cells will then be programmed to produce retroviral proteins and retroviral vector genomes at high levels. Since the adenovirus vectors are defective, no large amounts of direct cell lysis will occur and retroviral vectors can be harvested from the cell supernatants.

In an alternative system (which is more truly extracellular), the following components are used:

1. gag/pol and env proteins made in the baculovirus system in a similar manner as described in Smith et al. (supra) (or in other protein production systems, such as yeast or E. coli).

those preselected target cells.

VIII. Site-Specific Integration

Targeting a retroviral vector to a predetermined locus on a chromosome increases the benefits of gene-delivery systems. A measure of safety is gained by direct integration to a "safe" spot on a chromosome, i.e., one that is proven to have no deleterious effects from the insertion of a vector. Another potential benefit is the ability to direct a gene to an "open" region of a chromosome, where its expression would be maximized. Two techniques for integrating retroviruses at specific sites are described below.

(i) Homologous Recombination

One technique for integrating an exogenous gene of a vector construct of a recombinant retrovirus into a specific site in a target cell's DNA employs homologous recombination. Plasmids containing sequences of DNA of greater than about 300 bp that are homologous to genomic sequences have been shown to interact (either by replacement or insertion) with those genomic sequences at a rate that is greater than 10^2-fold over a specific interaction in the absence of such homology (see Thomas and Capecchi, Cell 51:503-12, 1987, and Doetscheman et al., Nature 330:576-78, 1987). It has been shown that an insertion even, or alternatively, a replacement event, may be driven by the specific design of the vector.

In order to employ homologous recombination in site-specific retroviral integration, a vector construct should be modified such that (a) homologous sequences (to the target cell's genome) are incorporated into the construct at an appropriate location; and (b) the normal mechanism of integration does not interfere with the targeting occurring due to homologous sequences. A preferred approach in this regard is to add homologous sequences (greater than about 300 bp) in the 3' LTR, downstream from the U3 inverted repeat. In this approach, the construct is initially made with a region of homology inserted in the 3' LTR at the Nhe 1 site in U3. Reverse transcription in the host cell will result in a duplication of the region of homology in the 5' LTR within 31 bp of the end of the inverted repeat (IR). Integration into the host genome will occur in the presence or absence of the normal integration mechanism. The gene in the vector may be expressed, whether from the LTR or from an internal promoter. This approach has the effect of placing a region of homology near a potential free end of the double-stranded retrovirus vector genome. Free ends are known to increase the frequency of homologous recombination by a factor of approximately 10. In this approach, it may be necessary to defeat the normal mechanism of integration, or to at least modify it to slow down the process, allowing time for homologous DNAs to line up. Whether this latter modification is required in a particular case can be readily ascertained by one skilled in the art.

(ii) Integrase Modification

Another technique for integrating a vector construct into specific, preselected sites of a target cell's genome involves integrase modification.

The retrovirus pol gene product is generally processed into four parts: (i) a protease which processes the viral gag and pol products; (ii) the reverse transcriptase; and (iii) RNase H, which degrades RNA of an RNA:DNA duplex; and (iv) the endonuclease or "integrase."

The general integrase structure has been analyzed by Johnson et al. (Proc. Natl. Acad. Sci. USA 83:7648-7652, 1986). It has been proposed that this protein has a zinc binding finger with which it interacts with the host DNA before integrating the retroviral sequences.

In other proteins, such "fingers" allow the protein to bind to DNA at particular sequences. One illustrative example is the steroid receptors. In this case, one can make the estrogen receptor, responding to estrogens, have the effect of a glucocorticoid receptor, responding to glucocorticoids, simply by substituting the glucocorticoid receptor "finger" (i.e., DNA binding segment) in place of the estrogen receptor finger segment in the estrogen receptor gene. In this example, the position in the genome to which the proteins are targeted has been changed. Such directing sequences can also be substituted into the integrase gene in place of the present zinc finger. For instance, the segment coding for the DNA binding region of the human estrogen receptor gene may be substituted in place of the DNA binding region of the integrase in a packaging genome. Initially, specific integration would be tested by means of an in vitro integration system (Brown et al., Cell 29:347-356, 1987). To confirm that the specificity would be seen in vivo, this packaging genome is used to make infectious vector particles, and infection of and integration into estrogen-sensitive and estrogen-nonsensitive cells compared in culture.

Through use of this technique, incoming viral vectors may be directed to integrate into preselected sites on the target cell's genome, dictated by the genome-binding properties of site-specific DNA-binding protein segments spliced into the in-

Production of env Proteins. Hybrid Envelope Proteins Using Housekeeping Promoters for Ubiquitous Expression in Transgenic Animals

This example utilizes the HPRT promoter for expression of either envelope or hybrid envelope proteins. The envelope proteins could be from any retrovirus that is capable of complementing the relevant gag-pol, in this case that of MLV. Examples are ecotropic MLV, amphotropic MLV, xenotropic MLV, polytropic MLV, or hybrid envelopes. As above, the envelope gene would be cloned behind the HPRT promoter using recombinant DNA techniques (see Maniatis et al., op. cit.). The resulting "minigene" would be isolated (see Hogan et al., op. cit.), and expression of envelope protein would be determined (Harlow et al., op. cit.). The transgenic envelope animals would be bred to homozygosity to establish a well-characterized envelope animal.

EXAMPLE 11

Production of gag-pol-env Animals Using Housekeeping Promoters for Ubiquitous Expression in Transgenic Animals

This would use the well-characterized gag-pol animals, as well as the animals for the establishment of a permanent gag-pol-envelope animal line. This would involve breeding to homozygosity and the establishment of a well-characterized line. These lines would then be used to establish primary mouse embryo lines that could be used for packaging vectors in tissue culture. Furthermore, animals containing the retroviral vector could be bred into this line.

EXAMPLE 12

Production of Tissue-Specific Expression of gag-pol-env or Hybrid Envelope in Transgenic Animals

The example given here is to direct tissue expression of the gagpol, envelope, or hybrid envelope to specific tissues, such as T-cells. This involves the use of CD2 sequences (see Lang et al., EMBO J. 7:1675-1682, 1988) that give position and copy number independence. The 1.5 kb BamH1 Hind III fragment from the CD2 gene would be inserted in front of gag-pol, envelope, or hybrid envelope fragments using recombinant DNA techniques. These genes would be inserted into fertilized mouse eva by microinjection. Transgenic animals would be characterized as before. Expression in T-cells would be established. Animals would be bred to homozygosity to establish well-characterized lines of transgenic animals. Gag-pol animals would be mated to envelope animals to establish gag-pol-env animals expressing only in T-cells. The T-cells of these animals would then be a source for T-cells capable of packaging retroviral vectors. Again, vector animals could be bred into these gag-pol-env animals to establish T-cells expressing the vector.

This technique allows the use of other tissue-specific promoters, such as milk-specific (whey), pancreatic (insulin or elastase), or neuronal (myelin basic protein) promoters. Through the use of promoters, such as milk-specific promoters, recombinant retroviruses may be isolated directly from the biological fluid of the progeny.

EXAMPLE 13

Production of Either Housekeeping or Tissue-Specific Retroviral Vectors in Transgenic Animals

The insertion of retroviruses or retroviral vectors into the germ line of transgenic animals results in little or no expression. This effect, described by Jaenisch (see Jahner et al., Nature 298:623-628, 1982), is attributed to methylation of 5' retroviral LTR sequences. This technique would overcome the methylation effect by substituting either a housekeeping or tissue-specific promoter to express the retroviral vector:retrovirus. The U3 region of the 5' LTR, which contains the enhancer elements, is replaced with regulatory sequences from housekeeping or tissue-specific promoters (see Figure 20). The 3' LTR is fully retained, as it contains sequences necessary for polyadenylation of the viral RNA and integration. As the result of unique properties of retroviral replication, the U3 region of the 5' LTR of the integrated provirus is generated by the U3 region of the 3' LTR of the infecting virus. Hence, the 3' is necessary, while the 5' U3 is dispensable. Substitution of the 5' LTR U3 sequences with promoters and insertion into the germ line of transgenic animals results in lines of animals capable of producing retroviral vector transcripts. These animals would then be mated to gag-pol-env animals to generate retroviral-produc-

24. The recombinant retrovirus of claim 9 wherein the vector construct expresses a reporting product on the surface of target cells infected with the retrovirus and containing the pathogenic agent.

25. The recombinant retrovirus of claim 1 wherein the vector construct directs the expression of a gene capable of suppressing the immune system in target cells infected with said retrovirus.

26. The recombinant retrovirus of claim 1 wherein the vector construct directs the expression of a blocking element in cells infected with said retrovirus, said blocking element being capable of binding to either a receptor or an agent such that the receptor/agent interaction is blocked.

27. The recombinant retrovirus of claim 1 wherein the vector construct directs the expression of a blocking element in cells infected with said retrovirus, said blocking element being capable of binding to a receptor or an envelope protein, such that the receptor/envelope protein interaction is blocked.

28. The recombinant retrovirus of claims 26 or 27 wherein the blocking element is secreted from the infected cells.

29. The recombinant retrovirus of any of claims 1-28, wherein said retrovirus is replication defective.

30. A method of producing a recombinant retrovirus, comprising:
packaging a vector construct in a capsid and envelope such that a replication defective recombinant retrovirus according to claim 29 is produced.

31. Ex vivo cells infected with a recombinant retrovirus according to any of claims 1-29.

32. Eucaryotic cells infected with a recombinant retrovirus according to any of claims 1-28, said cells being capable of generating infectious particles containing any one of said vector constructs.

33. A pharmaceutical composition comprising a retrovirus according to any one of claims 1-29, in combination with a physiologically acceptable carrier or diluent.

34. The pharmaceutical composition of claim 33, for use as an active therapeutic substance.

35. A method of testing a sample for the presence of a virus carrying a particular gene, said gene being capable of expressing an identifying protein in indicator cells having (1) a marker gene which is capable of expressing a marker product; and (2) control sequences which respond to the presence of the identifying protein by switching expression of the marker gene between expressing and non-expressing states;
the method comprising contacting indicator cells with the sample, and testing for the presence of the marker product.

36. A method of testing for the presence of a particular gene in a cell sample, said gene being capable of expressing an identifying protein, comprising:
infecting cells within the sample with a recombinant retrovirus comprising a vector construct coding for (1) a marker gene which is capable of expressing a marker product in the cell sample; and (2) control sequences which respond to the presence of the identifying protein in the cells within the sample by switching expression of the marker gene between expressing and non-expressing states; and
testing for the presence of the marker product.

37. The method of claims 35 or 36 wherein the control sequences switch expression of the marker product by switching the marker gene between transcribing and nontranscribing states.

38. An indicator cell suitable for testing for the presence of a virus carrying a particular gene which is capable of expressing an identifying protein in the indicator cell, the indicator cell having a genome coding for (1) a marker gene which can express a marker product in the indicator cell; and (2) control sequences which respond to the presence of the identifying protein in the indicator cell by switching expression of the marker gene between expressing and non-expressing states.

39. A recombinant retrovirus suitable for infecting cells and testing for the presence of a particular gene therein which produces an identifying protein, the recombinant retrovirus having a genome coding for:

(1) a marker gene which can express a marker product in the cells; and

(2) control sequences which respond to the presence of the identifying protein in the cells by switching expression of the marker gene between expressing and non-expressing states.

40. A method of selecting packaging cells which produce high levels of a primary agent selected from a packaging protein and a gene product of interest, comprising:

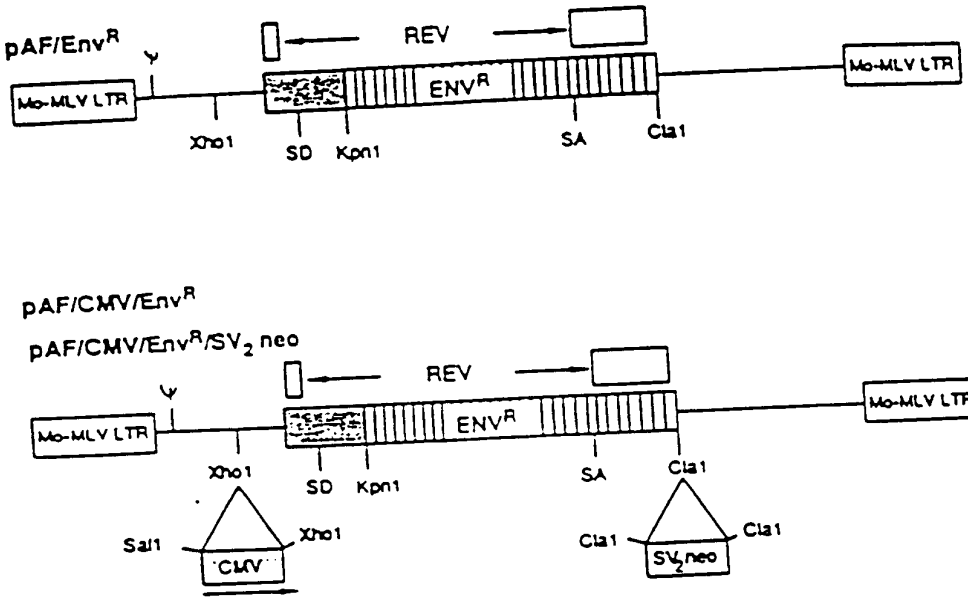
(a) providing in packaging cells a genome comprising a primary gene which expresses a primary agent therein, and a selectable gene which expresses a selectable protein therein at lower levels than the primary agent, the expression levels of the primary gene and selectable gene being proportional;

(b) exposing the packaging cells to a selecting agent which enables identification of those cells which express the selectable protein at a critical level; and

(c) detecting those packaging cells which express high levels of the primary agent.

RETROVIRAL CONSTRUCTS OF ENV^R

FIG.1



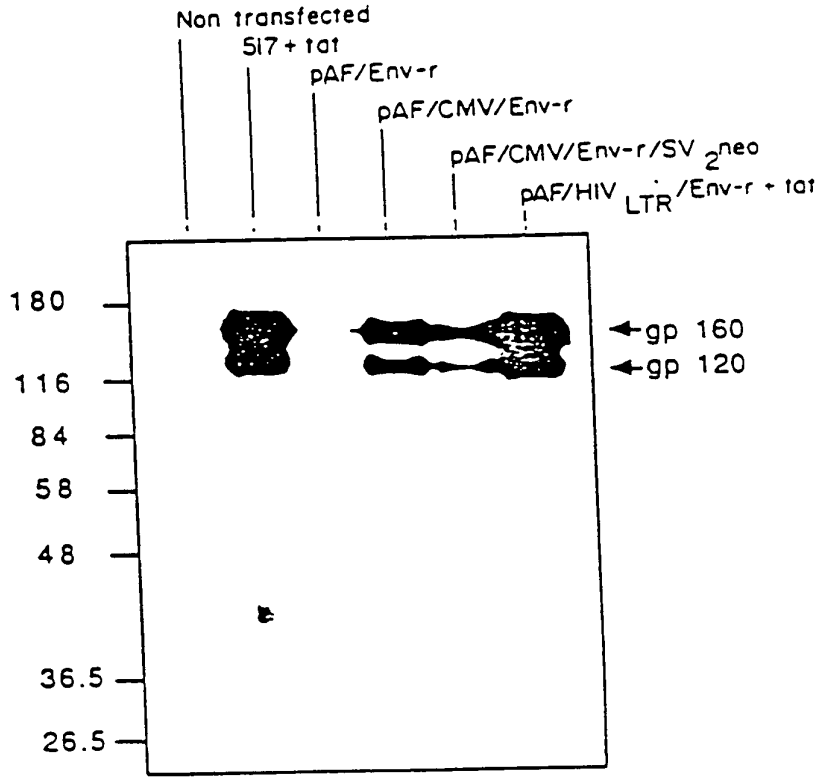
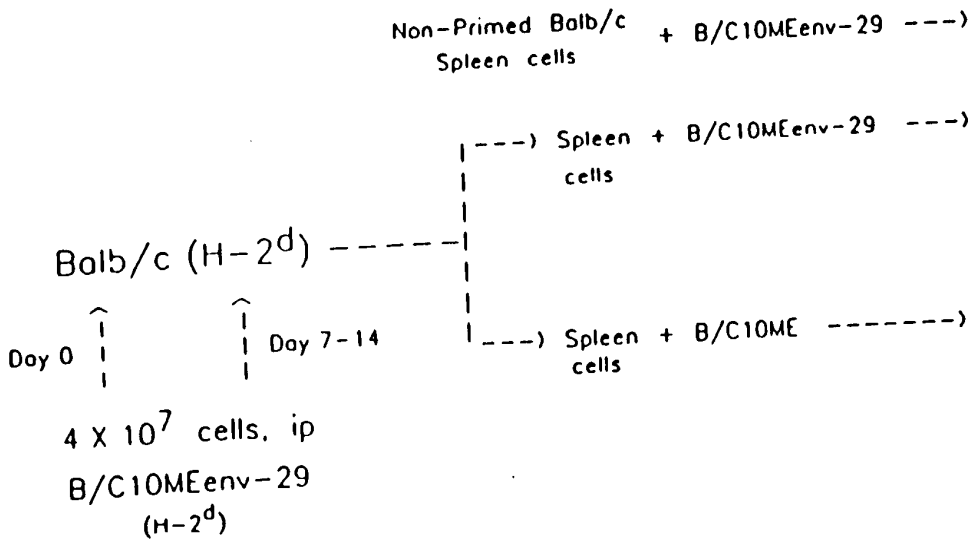


FIG.2

Induction of Anti-HIV env CTL in Balb/c Mice
Using Retroviral-Infected Stimulator Cells

FIG. 3



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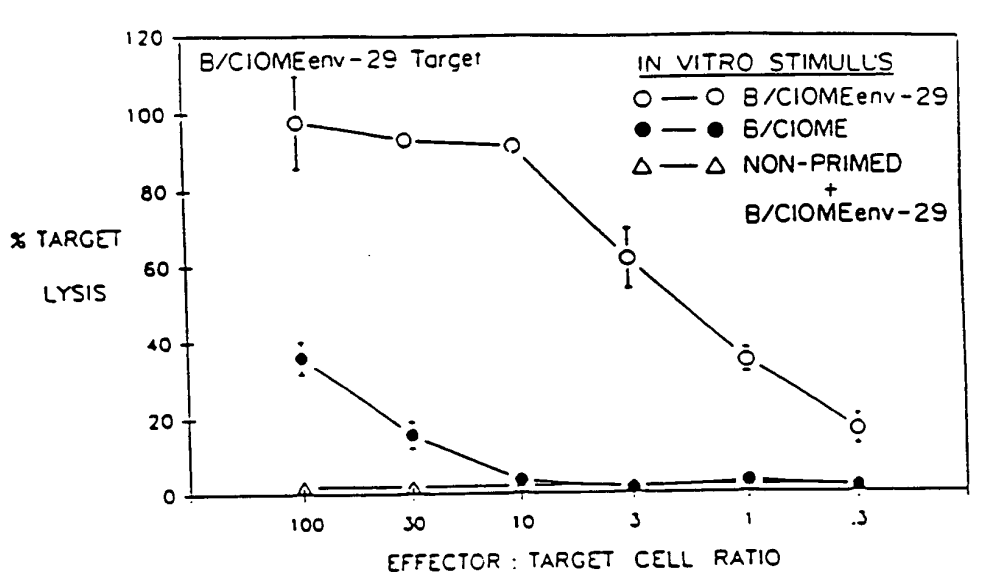


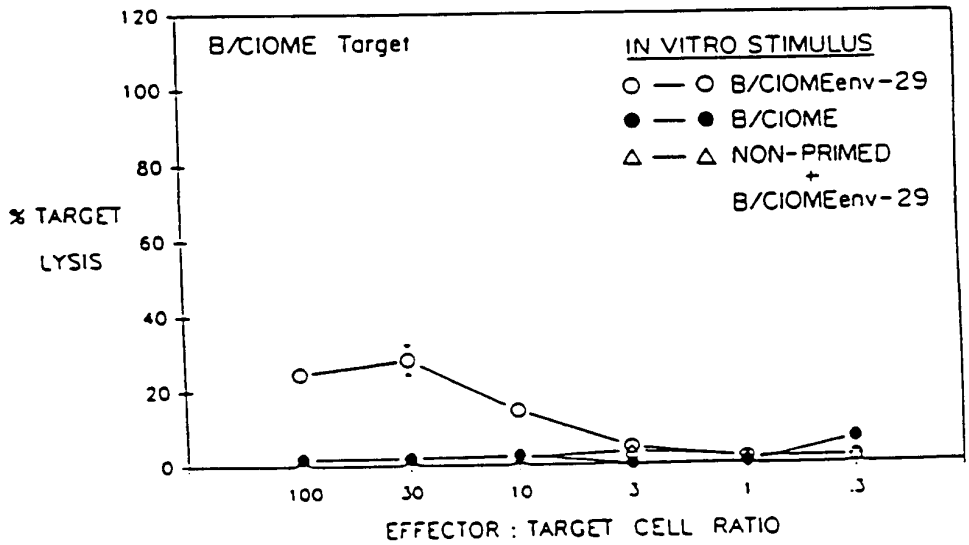


FIG. 4

RECEPTOR BLOCKER VECTOR

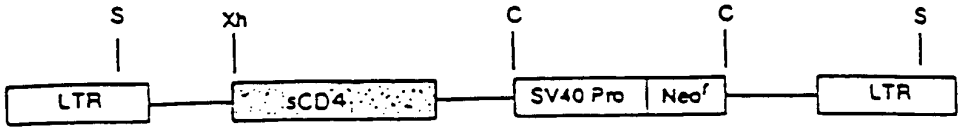


FIG.5

Construction of retroviral vectors pTK - 1 and pTK - 3

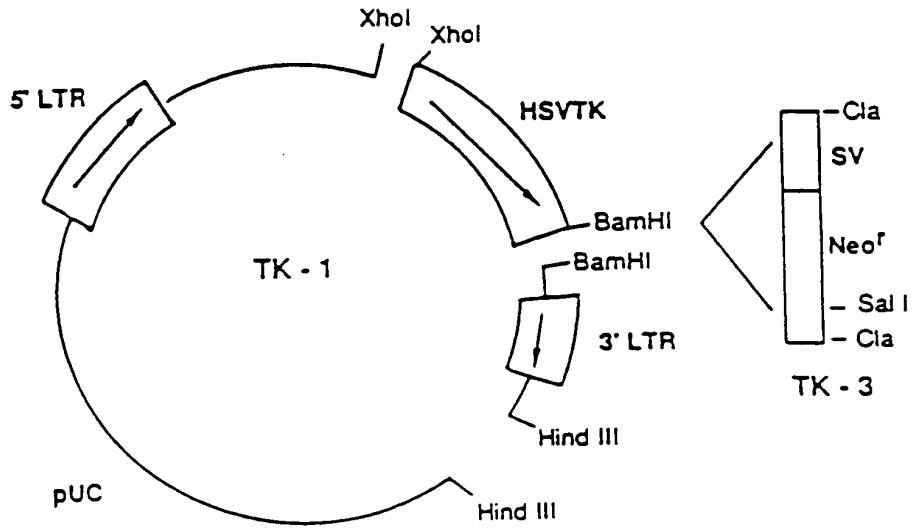


FIG.6

Construction of HIV-conditionally - lethal vector KTVIHAX

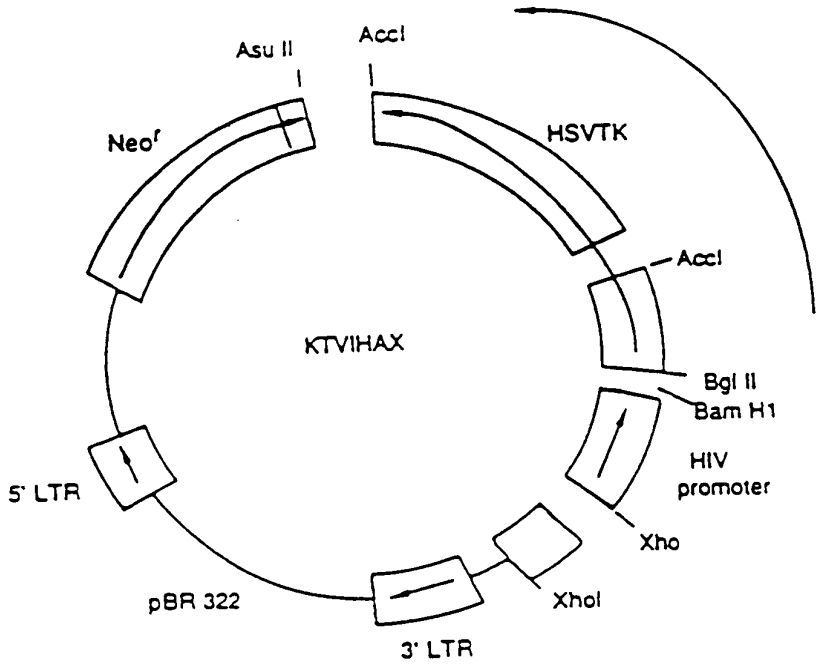


FIG. 7

Construction of KTVIH5 and KTVIH Neo

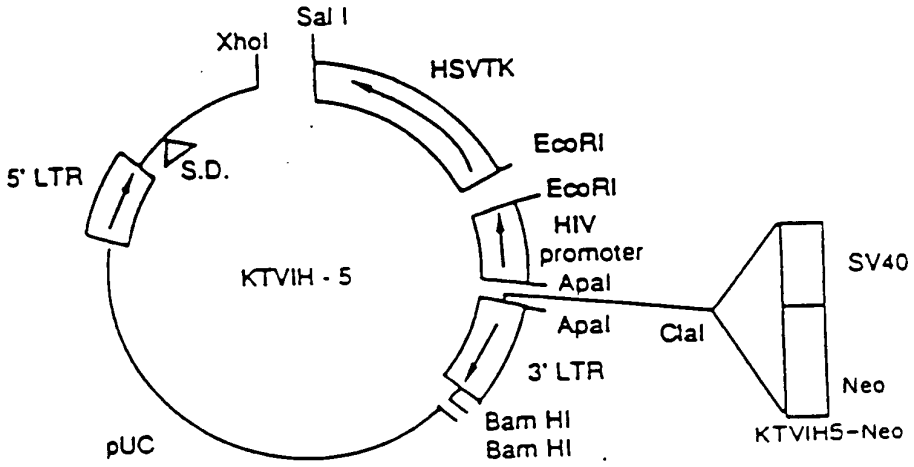


FIG. 8

CONSTRUCTION OF MHMTKNEO RETROVIRAL VECTOR

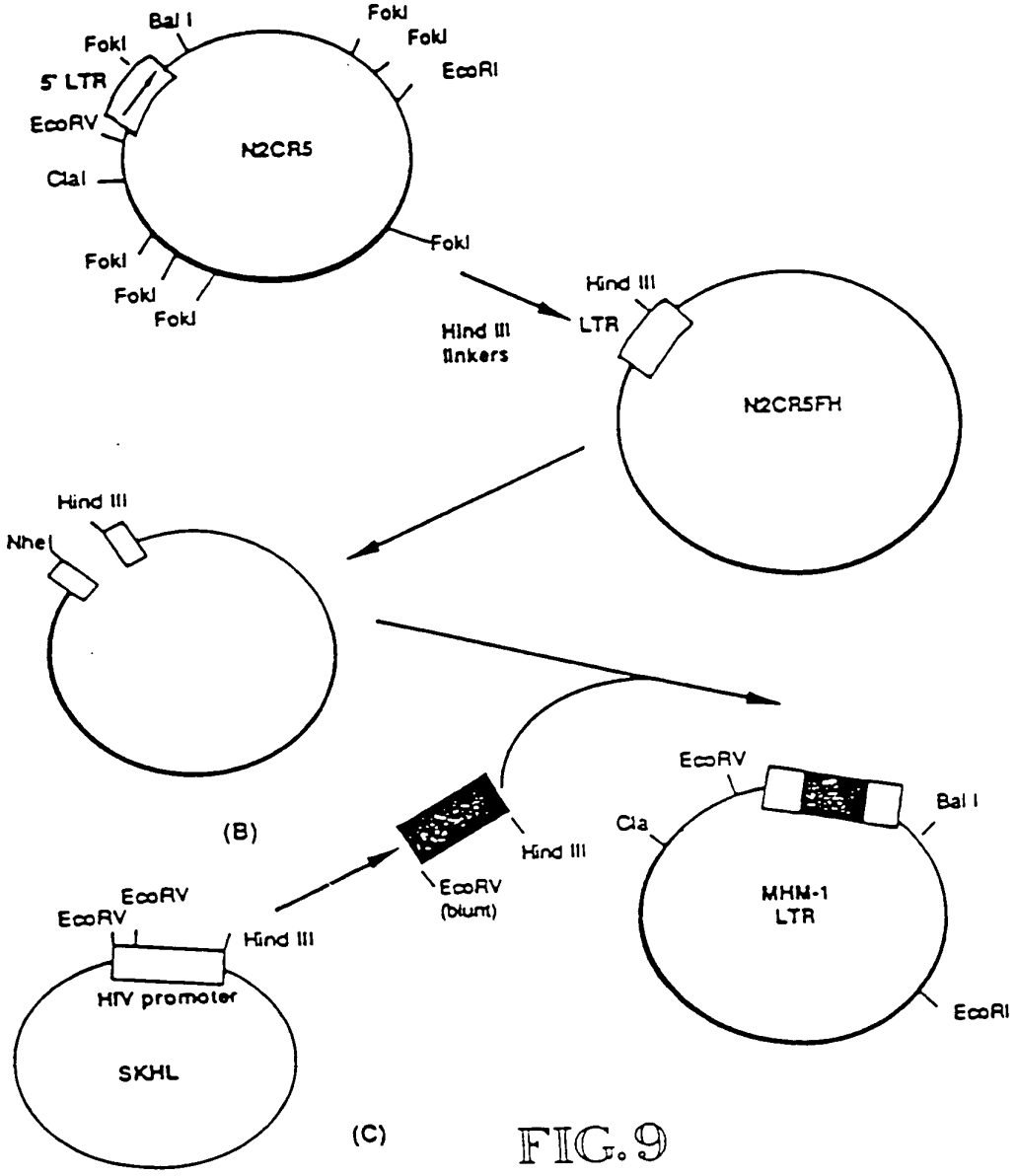
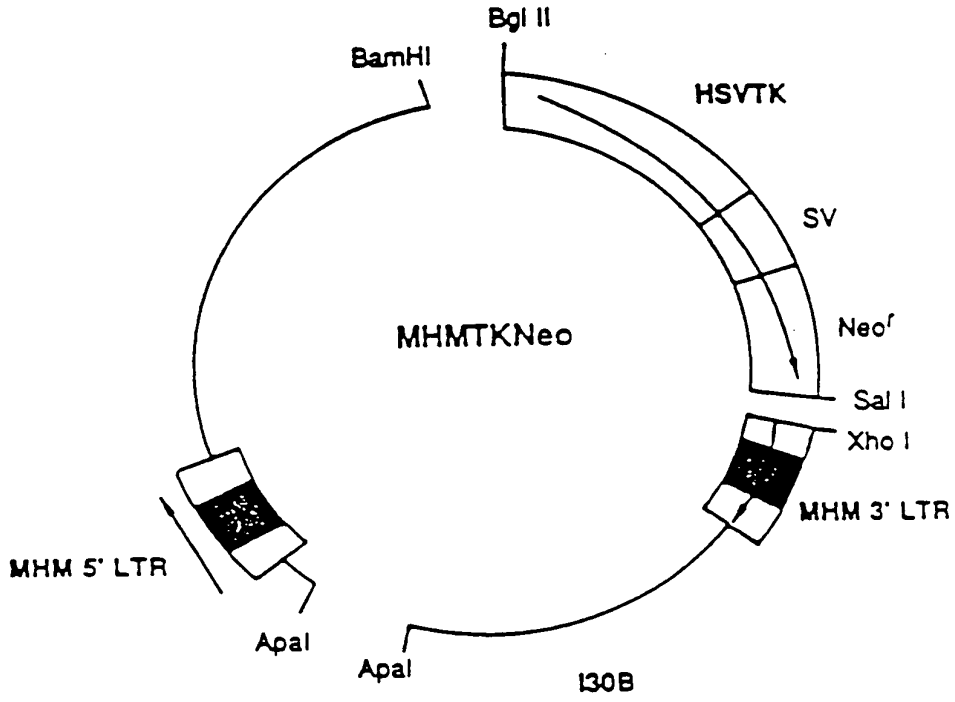


FIG. 9

FIG. 9 CONT.



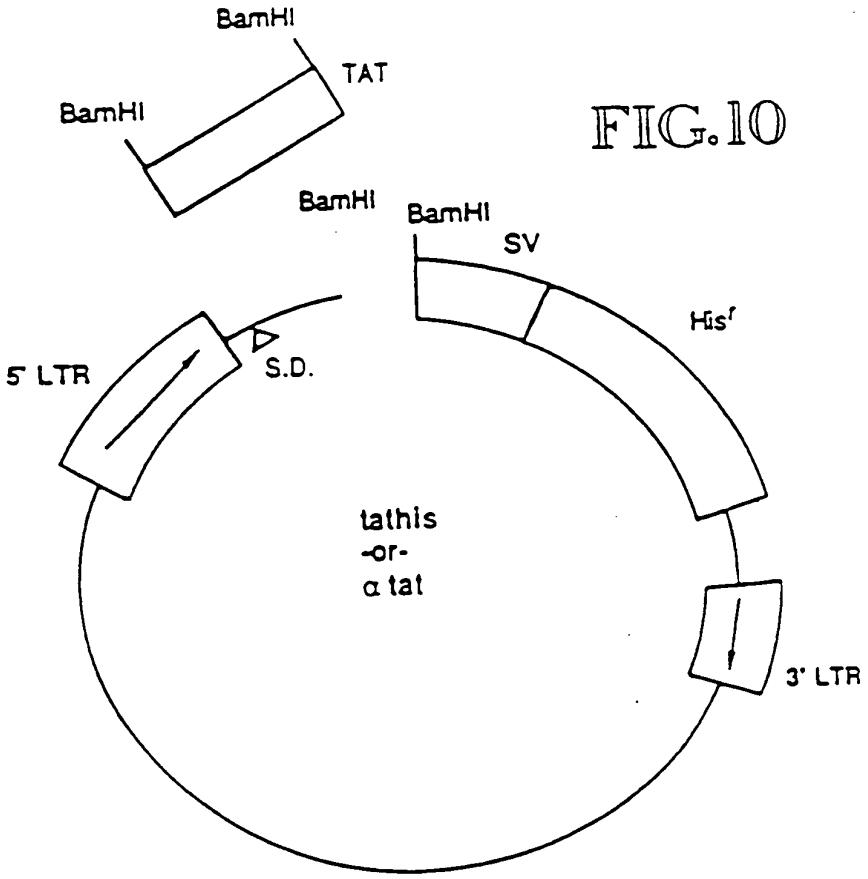


FIG.10

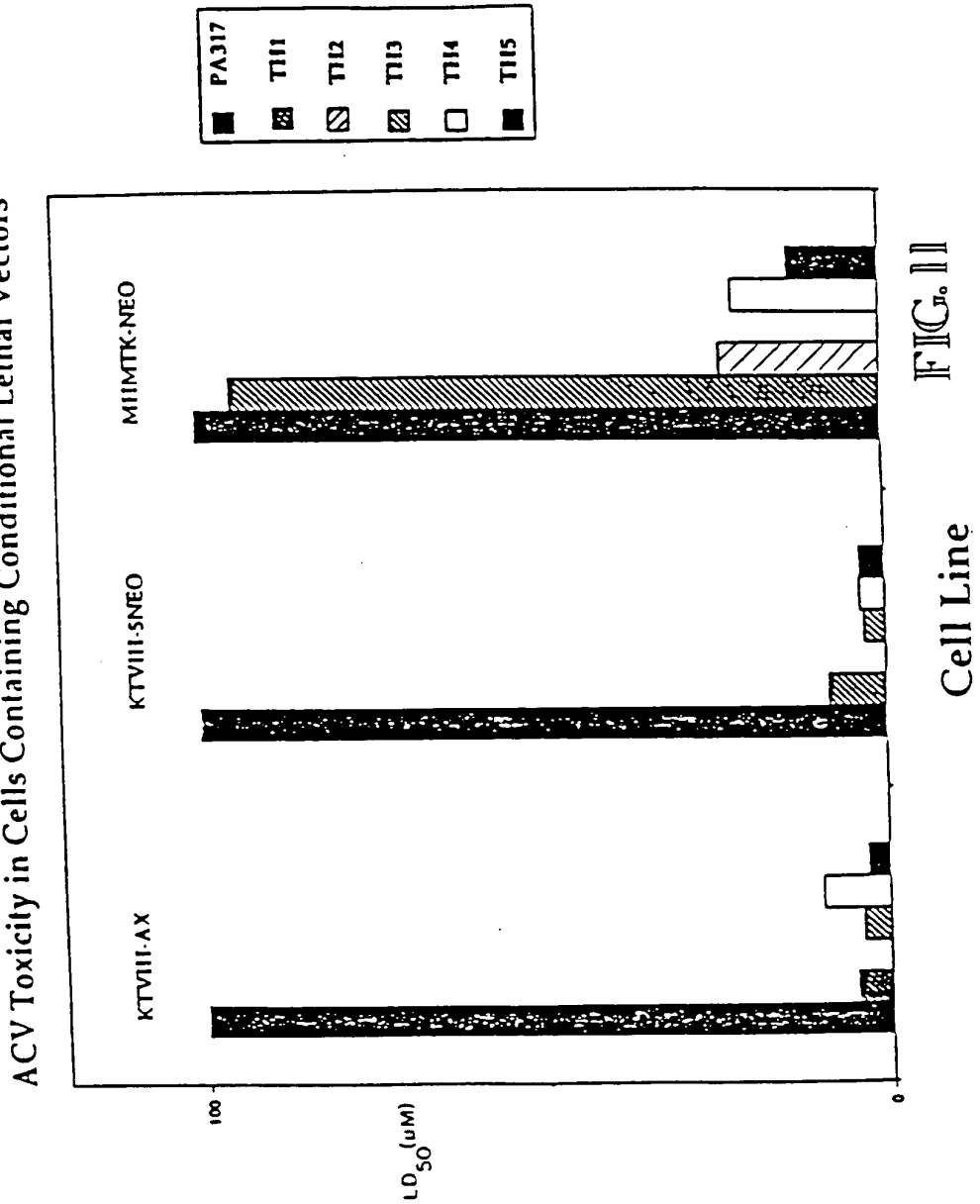


FIG. 11

FIG. 12

FIG. 13

EFFECT OF AZT ON HIV INFECTION

FIG. 14

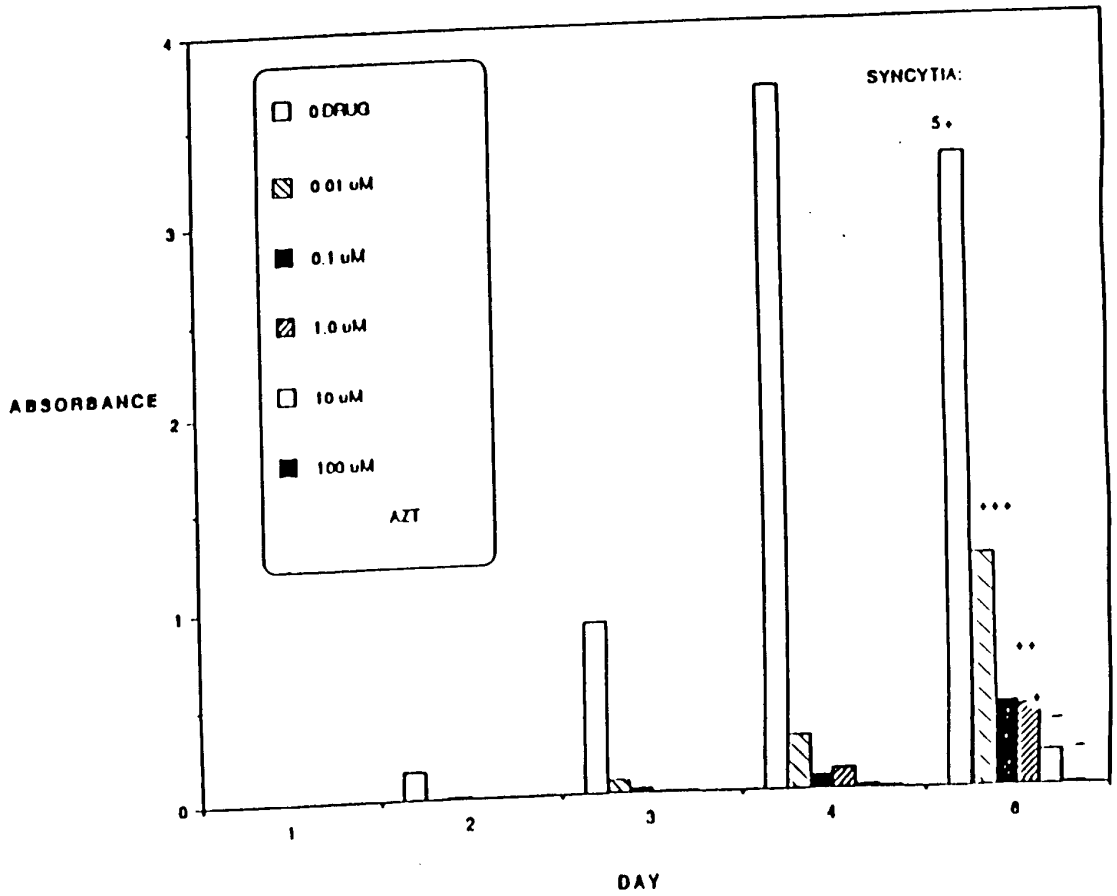


FIG. 15

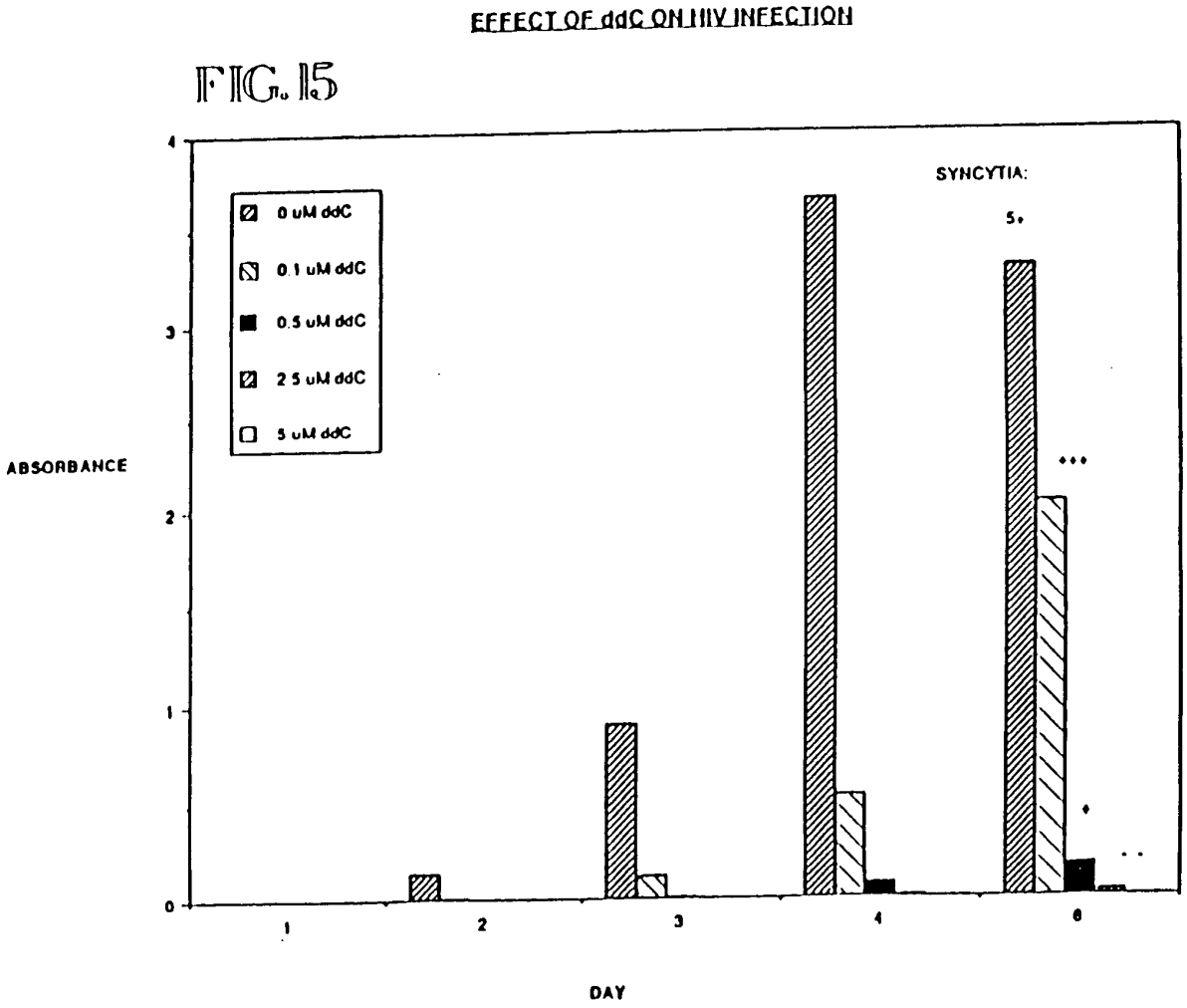
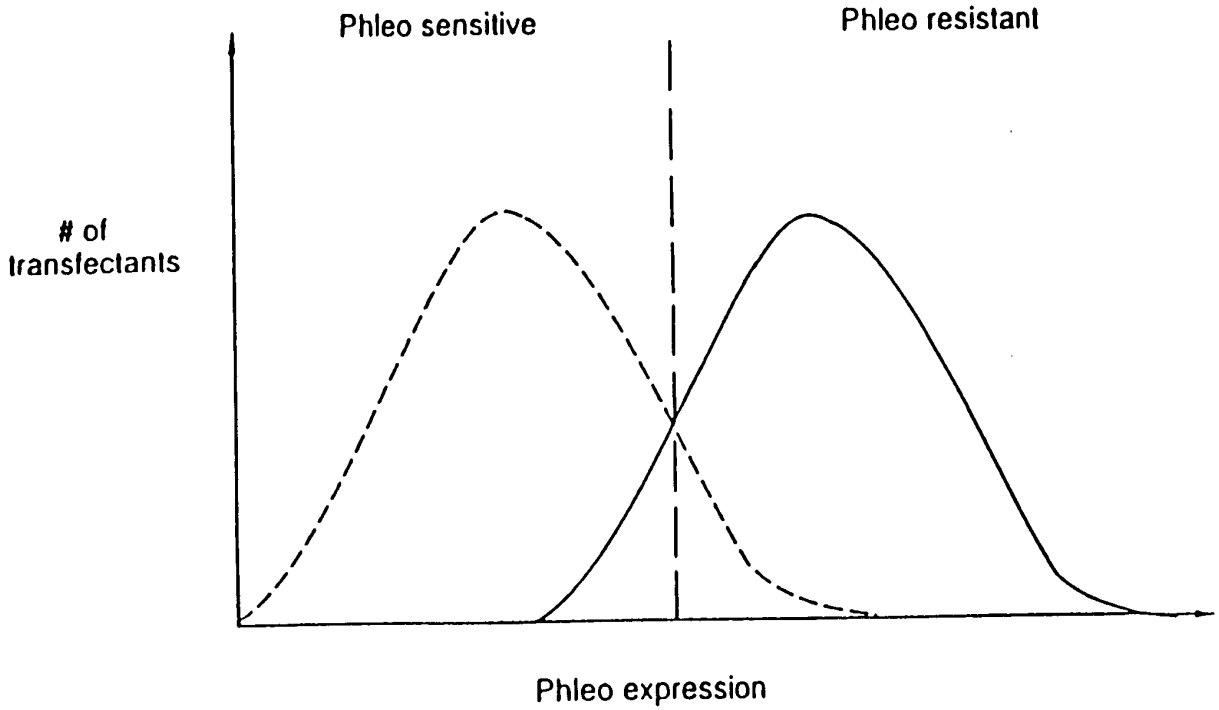

EFFECT OF ddC ON HIV INFECTION
SYNCYTIA:
5+
+++
+
0 uM ddC
0.1 uM ddC
0.5 uM ddC
2.5 uM ddC
5 uM ddC
ABSORBANCE
0
1
2
3
4
1
2
3
4
6
DAY

FIG. 16


Phleo sensitive
Phleo resistant
# of transfectants
Phleo expression

Plasmids Designed to Increase Viral Protein Production

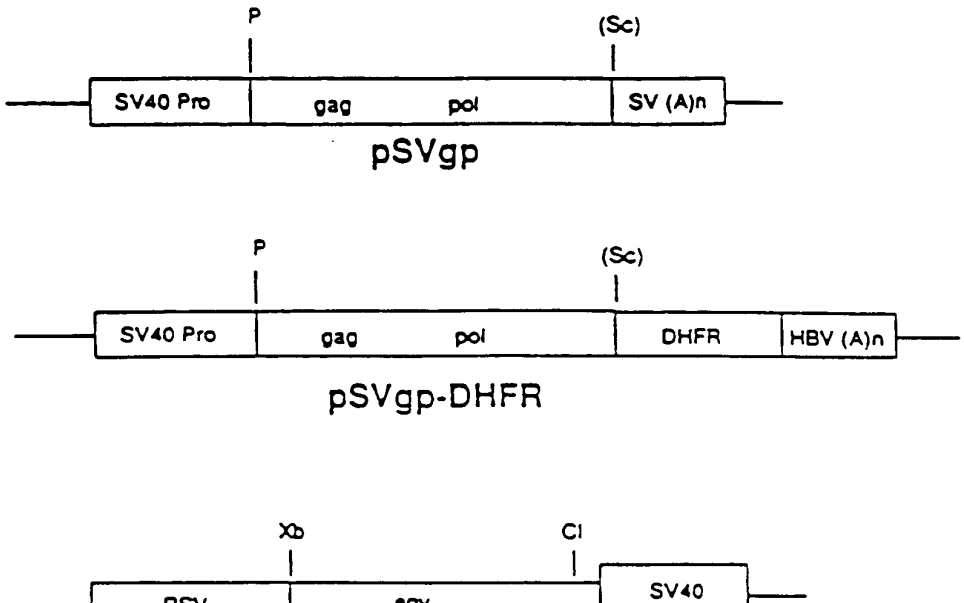
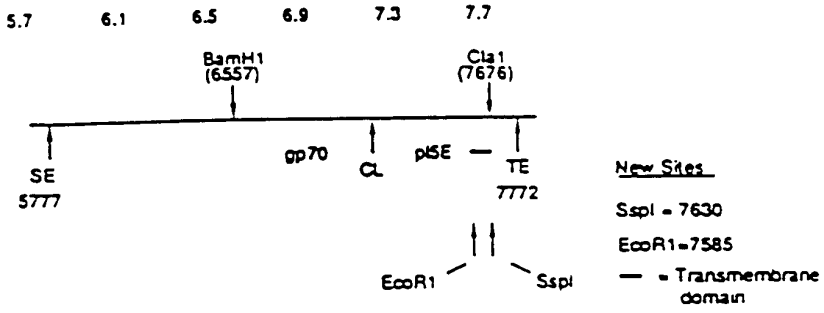


FIG. 17

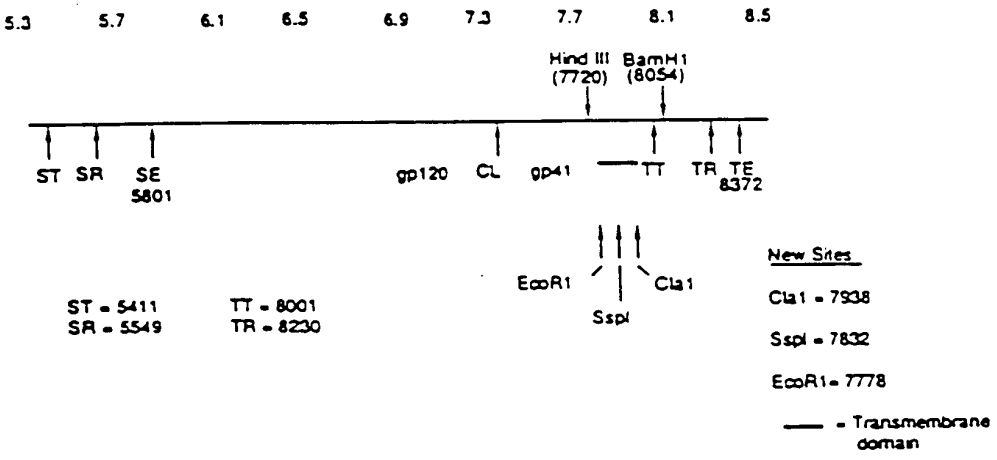
FIG. 18

CREATION OF FUSION SITES ON MLV AND HIV ENV GENES

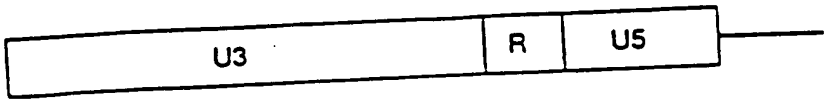
MLV



HIV



A. Normal 5' LTR



B. Hybrid 5' LTR

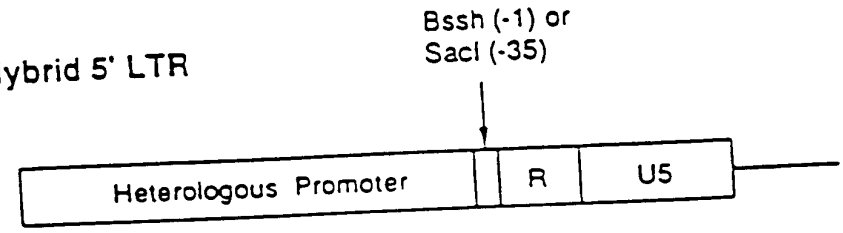
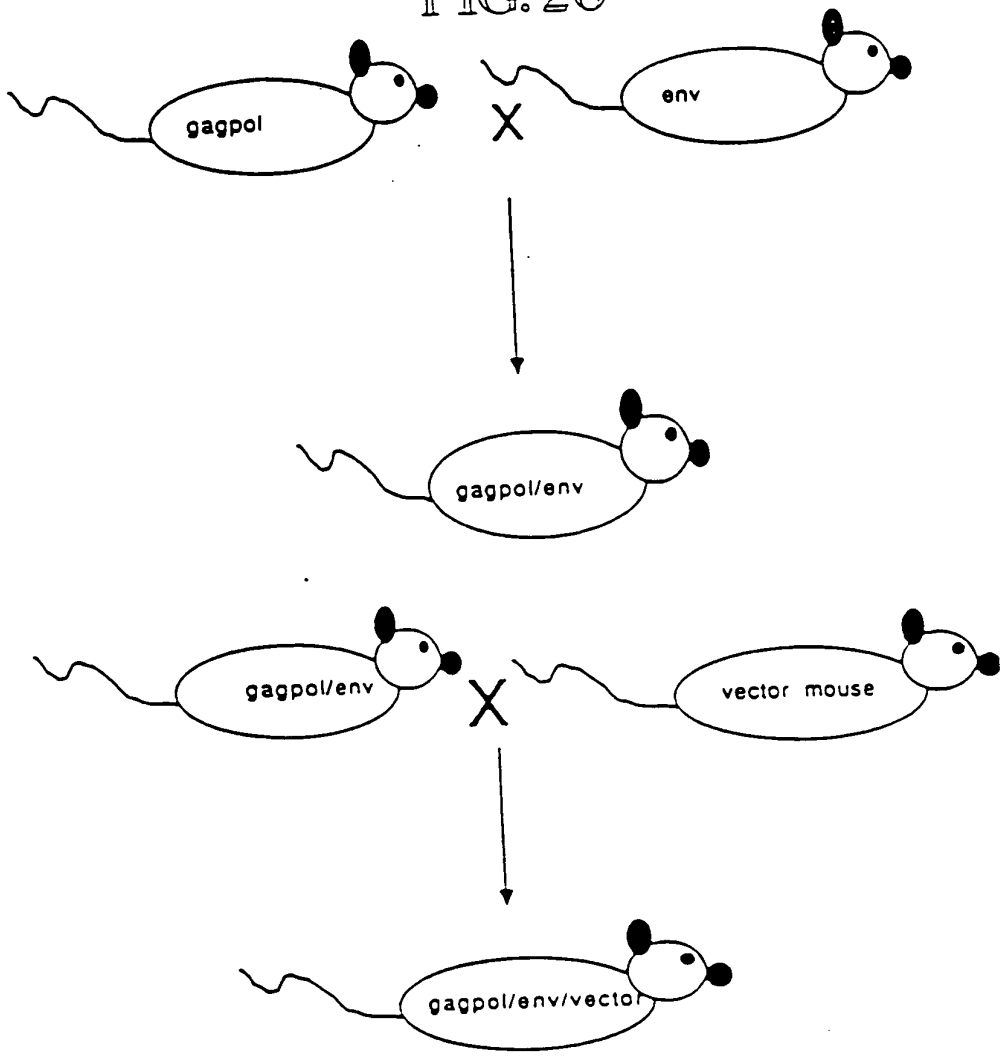


FIG. 19

FIG. 20



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Application Number

EP 89 10 5059

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X,D	EP-A-0 243 204 (CETUS CORP.) * Whole document *	1-6,8-13,19,29-34	C 12 N 15/00 C 12 N 7/00 C 12 N 5/00 A 61 K 35/76 C 12 Q 1/70
X	SCIENCE, vol. 229, 26th July 1985, pages 345-352; J.G. IZANT et al.: "Constitutive and conditional suppression of exogenous and endogenous genes by anti-sense RNA" * Figure 1 *	1-6,8-10,15,18,29-34	
X,D	NATURE, vol. 318, 5th December 1985, page 414; R. TELLIER et al.: "New strategies for AIDS therapy and prophylaxis" * Whole article *	1-6,8-10,15,29-34	
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 83, July 1986, pages 4794-4798; J.T. HOLT et al.: "Inducible production of c-fos antisense RNA inhibits 3T3 cell proliferation" * Whole article *	1-6,8-11,15,29-34	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 N
X	FR-A-2 559 159 (INSERM) * Example V *	1-6,8-10,29-34	
X,P	FR-A-2 606 030 (INSTITUT PASTEUR) * Pages 8,9 *	1-7,9,10,29-34,40-47	

-/-

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	28-06-1989	CUPIDO M.

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	SCIENCE, vol. 239, 8th January 1988, pages 184-187; B.K. FELBER et al.: "A quantitative bioassay for HIV-1 based on trans-activation" * Whole article *	35-39	
X	BIOTECHNOLOGY, vol. 3, no. 8, August 1985, pages 689-693, New York, US; D. McCORMICK: "Human gene therapy: The first round" * Whole article *	40-51	
A	NATURE, vol. 331, no. 6151, 7th January 1988, pages 78-81, London, GB; R.E. HUSSEY et al.: "A soluble CD4 protein selectively inhibits HIV replication and syncytium formation" * Figure 1 *	8,22	
			TECHNICAL FIELDS SEARCHED (Int. Cl.4)

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